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Effectiveness of Cleaning and Disinfection Procedures on the Removal of Enterotoxigenic *Bacillus cereus* From Infant Feeding Bottles

NEIL J. ROWAN* AND JOHN G. ANDERSON

Department of Bioscience and Biotechnology, University of Strathclyde, Royal College Building, 204 George Street, Glasgow, Scotland

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ABSTRACT

Reconstituted infant milk formulas are considered a food class of high risk because of the susceptibility of the infant population to enteric bacterial pathogens, severe response to enterotoxins, and increased mortality. Twenty infant feeding bottles, contaminated with different levels of enterotoxigenic *Bacillus cereus,* were subjected in triplicate to a variety of commonly used cleaning and disinfecting procedures. Although thorough cleaning reduced microbial numbers, it did not remove all *B. cereus* present. Three commercially available disinfection procedures (i.e., one chemical and two thermal) successfully eliminated this organism when the level of contamination was $<10^5$ organisms ml^{-1}. However, the chemical disinfection method failed to eliminate enterotoxigenic *B. cereus* totally at potentially hazardous contamination levels (i.e., $\geq 10^5$ organisms ml^{-1}) that may be encountered under storage abuse conditions in the home.

Infant populations are highly vulnerable to foods contaminated at low levels with enteric bacterial pathogens and their toxins, in part because of their immature immune system *(13).* Infant foods have been implicated occasionally as the etiological agents in a number of food-related illnesses *(8, 15).* Numbers of *Salmonella* spp. in infant milk formulas (IMF) implicated in previous foodborne outbreaks were very low, e.g., in the 1985 UK outbreak only three *Salmonella ealing* cells kg^{-1} were present *(23).*

Bacillus cereus, an occasional contaminant of dried infant milks, tolerates adverse environmental conditions better than most other bacterial enteropathogens *(14).* Becker et al. *(3)* reported that 54% of 261 samples of infant food distributed in 17 countries were contaminated with diarrheagenic *B. cereus,* reaching levels of 0.3–600 viable cells g^{-1}. When samples contaminated with approximately 100 cells ml^{-1} were reconstituted and incubated at 27°C, levels of 10^5 ml^{-1} were reached in 7–9 h. Rowan et al. *(22)* demonstrated that hospital-prepared infant food may be contaminated with diarrheagenic *B. cereus* at levels above the Association of Dietetic Food Industries of the European Communities (IDAEC) proposed safety limit of 10^3 colony-forming units $(CFU)g^{-1}$. In general, growth of enteric microbial pathogens in unprocessed milk is inhibited by the antagonistic action of resident lactic acid bacteria (LAB) *(5, 18).* Because LAB do not survive the spray-drying process, however *(16, 17),* contaminating deleterious organisms may proliferate in reconstituted baby foods under improper storage conditions *(21–23).*

Although the infectious dose of *B. cereus* is considered to be 10^5–10^7 cells ml^{-1} *(10),* outbreaks of foodborne illness associated with infants have been attributed to consumption of foods containing 10^3–10^5 cells g^{-1} *(9).* Granum et al. *(11)* suggested that the food industry should be concerned with levels as low as 10^3–10^4 ml^{-1} of food because food intoxication may be caused by ingestion of *B. cereus* cells or spores that may subsequently form enterotoxins in the ileum. In Norway, *B. cereus* was the most common cause of foodborne outbreaks in 1990 *(1).*

Donovan *(6)* showed that the main source of *B. cereus* contamination of raw milk obtained from creameries was derived chiefly from emptied cans that were allowed to stand for long periods before washing. The author suggested that vegetative cells of *B. cereus* readily formed spores in thin films of diluted milk in the rinsed cans. Hypochlorite disinfectants (e.g., Milton solution) are inactivated by food debris, organic matter, and catonic detergents *(2);* Hobbs and Roberts *(12)* showed that traces of milk remaining in baby bottles after careless washing totally inactivated 200 ppm available chlorine (i.e., the maximum amount recommended for disinfection). Other researchers showed wide variations in *B. cereus* spore thermal resistance, e.g., $D_{100°C}$ values ranged from 0.3 to 11.2 min in milk *(14),* which may play an important role in determining the efficacy of many commercially available thermal baby bottle disinfection procedures.

The principle objectives of this research were to determine the efficacy of commonly used cleaning and disinfection procedures on the removal of enterotoxigenic *Bacillus cereus* from infant feeding bottles subjected to periods of storage abuse which may occur in the home.

MATERIALS AND METHODS

Bacterial culture. The efficacy of infant feeding bottle cleaning and disinfection procedures was evaluated by use of *B.*

* Author for correspondence. Tel: 0044 141 552 4400, ext. 2531; Fax: 0044 141 553 1161; E-mail: n.j.rowan@pop-hub.strath.ac.uk.

cereus NCTC 11145, a diarrheagenic strain obtained from the National Collection of Typed Cultures. The diarrheagenic capability of this strain was previously demonstrated in reconstituted IMF *(20),* in which enterotoxin produced was detected with the *B. cereus* enterotoxin–reverse passive latex agglutination (BCET-RPLA) assay kit (Oxoid). Cultures were grown at 30°C and maintained on nutrient agar no. 2 (Oxoid Product); they were subcultured every 2 wk.

Infant feeding bottle cleaning procedures. Twenty precleaned and sterilized infant feeding bottles containing 250 ml of milk-based infant formula (in which 25 g of infant milk powder was reconstituted in 225 ml of sterile water at 56 ± 0.2°C, shaken 25 times through an excursion of 30 cm, and then tyndallized to sterility) were seeded with approximately 10^2 *B. cereus* spores ml^{-1} and then stored at 25°C for periods of up to 18 h (simulating storage abuse conditions that may occur in the home). Tyndallization consists of heating IMF on three successive days at 100°C for 30 min and leaving it at storage temperatures during the intervening times, during which spores can germinate so that resulting vegetative cells will be killed by the following period of heating at 100°C *(19).* The study was performed in triplicate. The starting inoculum was achieved by inoculating IMF with a spectrophotometrically (A_{600}) adjusted (Shimadzu uv-120-02) spore suspension prepared in phosphate-buffered saline (PBS; 0.01M; sodium phosphate, pH 7.2; 0.15M NaCl), in which *B. cereus* had been harvested from a 72-h culture grown at 30°C on nutrient agar no. 2 supplemented with 0.5 mg of $MnSo_4H_2O$ l^{-1} to aid sporulation (NMSA). Before IMF inoculation, the PBS cell/spore suspension was heat-treated in a water bath (Techne Tempette Junior TE-8J) at 65°C for 30 min to eliminate vegetative cells. Microbial numbers in the feeding bottles were confirmed by plating (1:10) dilutions onto NMSA plates that were then incubated at 30°C for 48 h.

Simulating the range of "after-use" situations that may be encountered in the home, the seeded baby bottles were subjected to the following range of commonly used handling procedures: (i) emptied of milk, (ii) emptied of milk and rinsed three times in warm water containing household detergent, and (iii) emptied of milk, washed, and brushed thoroughly in warm water containing household detergent (care was taken to ensure that all visible milk deposits were removed). In the subsequent text, baby bottlestreated with these procedures are referred to as uncleaned, partially cleaned, and thoroughly cleaned, respectively.

The extent to which *B. cereus* remained in these treated bottles was determined by filling each bottle with 250 ml of tyndallized IMF; the bottles were then shaken 25 times through an excursion of 30 cm before enumeration (to avoid ambiguity, IMF used as a bottle wash is referred to as IMF*). Microbial numbers were determined immediately after cleaning by spread and spiral plating (Spiral Plater Model B, Spiral Systems Inc.) duplicate samples of IMF* bottle wash onto *B. cereus* selective agar (BCSA). Plates were incubated aerobically at 25°C for 48–72 h. The identity of three representative cultures obtained on BCSA was confirmed by establishing the following morphological and biochemical properties: positive gram and catalase reactions, cell width (>1 μm) and length (>3 μm), motility and lecithovitellin/lecithinase production, β-haemolytic reaction and gross colony morphological appearance (millimeters), and an ellipsoidal endospore, centrally or subterminally positioned with nondistention of sporangium and characteristic API 50 CHB and API 20 E biochemical reaction profiles (Biomérieux Ltd.).

Infant feeding bottle disinfection methods. The following commercially available disinfection methods were tested: (i) feedtime steam sterilization (Boots), a thermal method in which feeding bottles were automatically steamed at 100°C for 15 min, (ii) microwave feedtime bottle steam sterilization (Boots), a thermal method in which bottles were placed in a sterilizing unit and steamed at 100°C in a microwave oven (Toshiba ER-686.E/EW) for 9 min, and (iii) complete baby feedtime sterilization (Boots), a chemical method in which bottles were immersed in 125 ppm sodium hypochlorite for 90 min. These methods are referred to as steam, microwave, and chemical, respectively, in Tables 1, 2, and 3.

These disinfection methods were carried out following details outlined in the manufacture's instructions. To examine the efficacy of each disinfection method, uncleaned, partially cleaned, and thoroughly cleaned feeding bottles containing different levels of *B. cereus* (described in the previous section) were subjected to each disinfection procedure. After disinfection, the bottles were filled with 250 ml of IMF* and incubated for periods of up to 18 h at 25°C. Microbial numbers in IMF* bottle wash were determined immediately after disinfection and after 14 and 18 h of incubation at 25°C.

TABLE 1. *Efficacy of different disinfection methods at eliminating enterotoxigenic* B. cereus *from untreated, partially cleaned, and thoroughly cleaned infant bottles in which the initial level of contamination was approximately 10^2 spores ml^{-1}*

	Disinfection method[a]								
	Steam			Microwave			Chemical		
Extent of cleaning	0 h	14 h	18 h[b]	0 h	14 h	18 h	0 h	14 h	18 h
Emptied	<1.0	<1.0	2.36[c]	<1.0	<1.0	1.85	<1.0	1.63	3.52
	—[d]	—	(1.28)	—	—	(0.87)	—	(0.73)	(1.49)
Emptied and rinsed	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	—	—	—	—	—	—	—	—	—
Emptied, washed, and brushed	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	—	—	—	—	—	—	—	—	—

[a] Microbial numbers in disinfected baby bottles were measured as $\log_{10}$ CFU ml^{-1}.

[b] Disinfected infant bottles, subjected previously to various cleaning regimes, were filled with 250 ml of IMF* and incubated for periods of up to 18 h at 25°C before enumeration.

[c] Mean *B. cereus* count for 20 feeding bottles analysed; the standard deviation is shown below the mean in parentheses. <1.0 refers to baby bottles having a *B. cereus* count below the lower detection limit of 1.0 $\log_{10}$ CFU ml^{-1}.

[d] —, Standard deviation of 0, in which all 20 bottles had *B. cereus* counts below the lower detection limit.

TABLE 2. *Efficacy of different disinfection methods at eliminating enterotoxigenic* B. cereus *from untreated, partially cleaned, and thoroughly cleaned infant feeding bottles that had been stored for 14 h at 25°C before disinfection*

	Disinfection method[a]								
	Steam			Microwave			Chemical		
Extent of cleaning	0 h	14 h	18 h[b]	0 h	14 h	18 h	0 h	14 h	18 h
Emptied	<1.0	2.64[c]	5.72	<1.0	2.40	5.58	2.49	4.79	6.52
	—[d]	(1.52)	(1.73)	—	(1.13)	(1.42)	(1.23)	(1.47)	(1.79)
Emptied and rinsed	<1.0	<1.0	3.74	<1.0	<1.0	3.57	<1.0	2.32	5.71
	—	—	(1.30)	—	—	(1.51)	—	(1.37)	(1.52)
Emptied, washed, and brushed	<1.0	<1.0	2.04	<1.0	<1.0	1.70	<1.0	1.13	3.64
	—	—	(0.83)	—	—	(1.23)	—	(0.47)	(1.32)

[a] Microbial numbers in disinfected baby bottles were measured as $\log_{10}$ CFU ml^{-1}.
[b] Disinfected infant bottles, subjected previously to various cleaning regimens, were filled with 250 ml of IMF* and incubated for periods of up to 18 h at 25°C before enumeration.
[c] Mean *B. cereus* count for 20 feeding bottles analysed; the standard deviation is shown below the mean in parentheses. <1.0 refers to baby bottles having a *B. cereus* count below the lower detection limit of 1.0 $\log_{10}$ CFU ml^{-1}.
[d] —, Standard deviation of 0, in which all 20 bottles had *B. cereus* counts below the lower detection limit.

TABLE 3. *Efficacy of different disinfection methods at eliminating enterotoxigenic* B. cereus *from untreated, partially cleaned, and thoroughly cleaned infant feeding bottles that had been stored for 18 h at 25°C before disinfection*

	Disinfection method[a]								
	Steam			Microwave			Chemical		
Extent of cleaning	0 h	14 h	18 h[b]	0 h	14 h	18 h	0 h	14 h	18 h
Emptied	<1.0	4.82	7.32[c]	<1.0	4.67	6.92	4.32	5.82	7.65
	—[d]	(1.15)	(1.92)	—	(1.47)	(1.79)	(1.30)	(1.82)	(1.63)
Emptied and rinsed	<1.0	2.60	5.20	<1.0	2.70	4.96	<1.0	4.93	6.79
	—	(1.57)	(1.57)	—	(0.95)	(1.82)	—	(1.51)	(1.79)
Emptied, washed, and brushed	<1.0	<1.0	4.18	<1.0	<1.0	3.92	<1.0	3.70	5.88
	—	—	(1.42)	—	—	(1.17)	—	(1.42)	(1.37)

[a] Microbial numbers in disinfected baby bottles were measured as $\log_{10}$ CFU ml^{-1}.
[b] Disinfected infant bottles, subjected previously to various cleaning regimens, were filled with 250 ml of IMF* and incubated for periods of up to 18 h at 25°C before enumeration.
[c] Mean *B. cereus* count for 20 feeding bottles analysed; the standard deviation is shown below the mean in parentheses. <1.0 refers to baby bottles having a *B. cereus* count below the lower detection limit of 1.0 $\log_{10}$ CFU ml^{-1}.
[d] —, Standard deviation of 0, in which all 20 bottles had *B. cereus* counts below the lower detection limit.

Statistical analysis. Fisher's exact test was used to compare microbial numbers in infant feeding bottles subjected to various cleaning and/or disinfecting procedures (Minitab Statistical Software Version 11; Minitab Plc.). All significant differences were reported at the 95% level of confidence ($P < 0.05$).

RESULTS AND DISCUSSION

Efficacy of commonly used infant feeding bottle cleaning procedures. The study showed that the greater the level of infant bottle cleaning, the larger the reduction in microbial numbers (Figure 1); the effectiveness of each cleaning stage at removing diarrheagenic *B. cereus* differed at the $P < 0.05$ level. Thorough cleaning of bottles contaminated with 10–100 spores ml^{-1}, a level of contamination occurring occasionally in IMF *(21)*, did not remove all of the spores present. Potentially hazardous levels of *B. cereus* remained in more heavily contaminated bottles (i.e., those in which the level of IMF contamination was approximately 10^5 organisms ml^{-1} after 14 h of storage) subjected to rigorous washing and brushing (Figure 1). Because thorough cleaning did not totally remove *B. cereus* from feeding

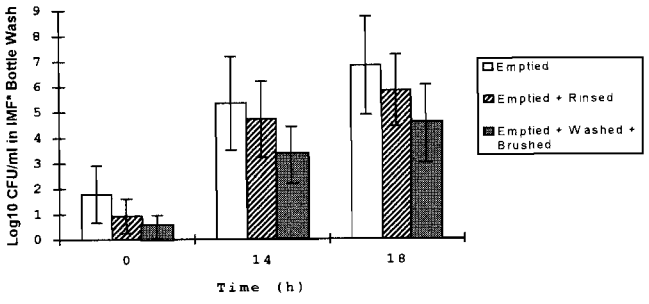

Figure 1. *Effect of extent of cleaning on the removal of enterotoxigenic* B. cereus *from infant feeding bottles stored for periods of up to 18 h at 25°C before treatment.* □, *Emptied;* ▨, *emptied and rinsed;* ■, *emptied, washed, and brushed.*

bottles (i.e., removal of visible milk deposits did not provide microbial free bottles), baby bottle cleaning must be supplemented with an appropriate disinfection method to provide safe food for infant consumption.

Efficacy of commercially available infant feeding bottle disinfection procedures. All disinfection methods successfully reduced diarrheagenic *B. cereus* to a nondetectable level (i.e., <10 CFU ml^{-1}) when the initial level of contamination was $\leq 10^5$ CFU ml^{-1} (Tables 1 and 2). In IMF* bottle wash, *B. cereus* emerged earlier (i.e., after 14 h of incubation at 25°C) in uncleaned baby bottles that had been subjected to the chemical disinfection method (Table 1). Application of both thermal disinfection methods did not totally eliminate *B. cereus;* reemergence was detected in IMF* after 18 h of incubation (Table 1). The level of contamination (i.e., period during which bottles were held under storage abuse) and the degree of bottle cleaning before disinfection affected the length of time that bottles remained with *B. cereus* at undetectable levels ($P < 0.05$); *B. cereus* emerged earlier in uncleaned bottles that had been stored for longer periods before disinfection (Tables 2 and 3).

The chemical method failed to disinfect uncleaned feeding bottles contaminated with approximately 10^5 organisms ml^{-1} (Tables 2 and 3). Subsequent storage of thermally disinfected bottles resulted in the detection of this organism at potentially hazardous levels in IMF* after 14 h of incubation at 25°C. Both steam disinfection procedures were equally efficient at removing *B. cereus* from baby bottles contaminated with $\geq 10^5$ CFU ml^{-1} ($P < 0.05$), and both methods were significantly better than the chemical method ($P < 0.05$) (Tables 2 and 3). Ayliffe et al. *(2)* reported the isolation of *Klebsiella aerogenes* and *Pseudomonas* spp. from baby bottles even after they had been thoroughly cleaned and disinfected in a sodium hypochlorite solution. The authors showed that this chemical disinfection process may occasionally be hazardous even when cleaning appeared to be efficient. Hypochlorite solution must be changed daily because the activity of this disinfection procedure diminishes with time *(2),* a requirement that may be prone to neglect.

This study has reaffirmed that infant bottle cleaning practices must be supplemented with a disinfection method to provide bottles free of potentially hazardous organisms. Although both heat and chemical disinfection procedures successfully removed *B. cereus* spores from contaminated baby bottles (at levels occurring occasionally in freshly prepared infant feeds), the latter method was inefficient at disinfecting uncleaned and partially cleaned bottles contaminated with the large numbers of this organism that occur during storage abuse. Failure to eliminate *B. cereus* will result in an increased inoculum and enhanced contamination of subsequently prepared infant feeds. Although *B. cereus* was the focus of this study, other *Bacillus* spp. (i.e., *B. licheniformis, B. subtilis, B. pumilus, B. brevis, B. thuringiensis,* and *B. sphaericus*) occurring occasionally in dried IMF products *(21)* have been implicated as the etiological agents in proven foodborne illness outbreaks *(16)* and opportunistic infections *(4, 7);* the majority of these organisms show greater heat resistance than *B. cereus (19).* Recent evidence has shown that IMF supplemented with maltodextrin stimulated growth of *B. cereus* and synthesis of diarrheal enterotoxins under improper storage conditions *(20);* therefore, extra care should be taken to ensure that baby bottles are thoroughly cleaned and disinfected before use.

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